RESEARCH ARTICLE

Exploring the impact of *Escherichia coli* outer membrane proteins on immune response and inflammation in rabbits

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Most Gram-negative bacteria have an outer cell membrane containing outer membrane proteins (OMPs) that are arranged parallel along the length of the cell. This study investigated the immunomodulatory effects of *Escherichia coli* OMPs in laboratory animals (rabbits). 60 matured male rabbits (*Oryctolagus cuniculus*) weighing approximately 1 kg each were used to isolate and characterize *E. Coli* OMPs. The rabbits were subsequently immunized with 0.1 mL *E. Coli* OMPs at a concentration of 1.7 mg/mL by subcutaneous injection. A polyacrylamide gel electrophoresis sodium dodecyl sulfate (PAGE-SDS) was employed for results evaluation and found only single band of OMP at 48 kDa that consistent with the expected size of a major *E. Coli* outer membrane protein. We hypothesized that the exposure to *E. Coli* OMPs would alter splenic function and rabbit cellular immune responses. The results showed that the spleen weight index was significantly higher in the OMP-exposed group than that in the control group ($P < 0.05$). Additionally, OMP exposure led to increased levels of interleukin-10 (IL-10) and interleukin-3 (IL-3) compared to controls. These findings elucidated the role of OMPs in triggering inflammatory responses and suggested their potential to induce both humoral and cellular immunity. This study showed important points about the complex interactions between OMPs and the immune system potentially paving the way for the development of novel therapeutic strategies against *E. Coli* infections.

Keywords: outer membrane protein; *E. coli*; IL-3; IL-10; SDS-PAGE; immunity.

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Introduction

Gram-negative bacteria have a distinct structure with two membranes that are an outer membrane (OM) encasing the inner cytoplasmic membrane, separated by a periplasmic space [1]. The OM harbors various proteins crucial for bacterial survival, pathogenicity, and defense against the host immune system [2, 3]. The channel-forming proteins facilitate nutrients and metabolites imported across the OM [4]. OMP synthesis occurs at the ribosomes and is facilitated by dedicated chaperones like SurA, Skp, and DegP, which escort them from the endoplasmic reticulum to the OM for insertion [5, 6]. Notably, the beta-barrel assembly machine (BAM) complex, a multiprotein machinery with BamA being a defining component specific to Gram-negative bacteria, assembles OMPs [7-9]. Other essential BAM proteins include BamB, BamC, BamD, BamE, and BamF [10].

Gram-negative bacteria shed spherical outer membrane vesicles (OMVs) containing OMPs, lipopolysaccharides (LPS), and other biomolecules [11]. These OMVs act as natural immunomodulators, stimulating both innate and adaptive immunity, making them attractive vaccine candidates due to their safety, immunogenicity, and ability to present multiple antigens [11]. OMPs display surface structures recognized by the host immune system as pathogen-associated molecular patterns (PAMPs) [12]. These ubiquitous proteins are crucial for maintaining bacterial cell shape and integrity and are characterized by an N-terminal signal sequence, a conserved C-terminal domain, and a signature beta-barrel fold [13-15]. The number of beta-strands in OMPs is often even, and some, like OmpX (binding protein) and Alts in *E. Coli* and *Yersinia pestis*, are associated with virulence [16-18].

Enterobacteriaceae, a family of Gram-negative bacteria, are known for secreting endotoxin (LPS) from their outer membrane. This potent inflammatory molecule plays a significant role in bacterial virulence [19, 20]. The interactions between LPS and OMPs with host immune cells have been extensively studied in animal models like rabbits, rats, and mice to understand their inflammatory potential [21, 22]. OMPs can stimulate both innate and adaptive immunity, making them ideal candidates for studying inflammatory responses *in vivo* and *in vitro* [23, 24]. Recent studies suggest the potential of OMPs for developing *E. Coli* vaccines [25-28]. *E. coli*, a Gram-negative bacterium responsible for diverse infections, utilizes OMPs for interaction with the host immune system. While OMPs are known to stimulate immune responses, their specific effects on cellular immunity and inflammatory markers like spleen weight remain unclear. This study investigated the immunomodulatory effects and inflammatory responses elicited by *E. coli* OMPs in rabbits and explored the impact of *E. coli* OMPs on spleen weight index and cellular immunity in rabbits, aiming to contribute to the development of OMP-based vaccines.

Materials and methods

Outer membrane protein (OMPs) preparation

Escherichia coli was obtained from a characterized culture collection maintained by the Department of Biology, University of Babylon, Babylon, Iraq. The bacteria were grown in 10 L of Luria-Bertani (LB) broth with agitation at 37°C until reaching the late log phase by monitoring the optical density (OD) at 280 nm. Cells were harvested by centrifugation at 5,000 rpm for 20 minutes at 4°C using Mikro 220R refrigerated centrifuge (Hettich, Iphofen, Germany). The cell pellet was resuspended in a buffer (10 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, pH 7.4) at a volume of 2.5 times the pellet's wet weight. Following incubation at 56°C for 1 h, cellular debris was removed by centrifugation at 18,000 rpm for 5 minutes after using a Mujin XHF-D disperser (Mujin, Ningbo, Zhejiang, China). The bacterial lysate was subjected to sequential centrifugation steps at 5,000 rpm for 20 minutes to remove unbroken cells followed by 30,000 rpm for 20 minutes to pellet the OMPs. The final OMP pellet was obtained by ultracentrifugation of the supernatant at 150,000 rpm for 2 hours at 4°C. The supernatant was discarded, and the OMP pellet was resuspended in phosphate-buffered saline (PBS) (Elabscience Co., Ltd., Shanghai, China). The purified OMPs in PBS were stored at -80°C until further use [29].

Determination of OMPs molecular weight by SDS- page

The molecular weight of the outer membrane protein (OMPs) was determined by using the Mini-Protean II electrophoresis (Bio-Rad Laboratories ltd, Hercules, California, USA) on a 5–20% polyacrylamide gradient gel and proteins were detected by staining with Coomassie brilliant blue [30].

Experimental animals

Sixty (60) adult male New Zealand White rabbits (*Oryctolagus cuniculus*) aged 3-5 months and weighed approximately 1 kg each were obtained from the Department of Biology, College of Sciences, University of Babylon, Babylon, Iraq

and used in this study to investigate the immune response to the antigen. Upon arrival, the animals were housed in dedicated cages within the institute's animal facility and allowed to acclimatize for two weeks. Throughout the experiment, the rabbits had free access to clean food and water to ensure their well-being [11]. All animal care and experimental procedures adhered strictly to the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the Council of University of Babylon, Hillah, Babylon city, Iraq.

Immune experimental animals

The influence of *E. Coli*-derived outer membrane vesicles (OMVs) on the spleen weight index and cellular immunity in rabbits were investigated. The animals were divided into two groups as 30 animals in group A, which were exposed to the antigen's outer membrane and 30 animals in group B as control. Both groups received 0.5 mL of Freund's incomplete adjuvant (oil), a water-in-oil emulsion that contains mineral oil along with emulsifiers, with 0.5 mL of 1.7 mg/mL outer membrane for group A and 0.5 mL of normal saline (0 mg/mL outer membrane) for group B in the first week. During the following 2 weeks, 1 mL per kg body weight of each OMPs or saline were injected to the experimental animals in each group.

Determination of spleen weight index

Garrido-Maestu method was employed to determine the spleen weight index. Animals were first anesthetized using chloroform, and then, the spleen was carefully dissected with the connective tissue being meticulously removed. The isolated spleen was then placed in a sterile petri dish containing PBS buffer. The spleen was gently dried on sterile blotting paper and the weight was measured to calculate the spleen weight index. The spleen weight index was expressed as the percentage of the spleen's weight relative to the total body weight, which provided valuable insights into the relationship between spleen size and overall body mass [30].

Skin test

In the fourth week of the study, each group of injected animals underwent the skin test. The animals in group A received an intradermal injection of 0.1 mL of mixture dose including 0.5 mL of outer membrane and 0.5 mL of oil. In contrast, the control animals (group B) were injected with an equivalent volume of normal saline. Skin changes were meticulously observed at 4, 24, 48, and 72 hours after the injections, and were compared between the groups to assess the impact of the outer membrane on eliciting the cellular response in rabbits [12].

Measurement of interleukin 10

The interleukin 10 (IL-10) was measured using the enzyme-linked immunosorbent assay (ELIZA) kit (Elabscience Co., Ltd., Shanghai, China) following the manufacturer's instructions. The absorbance was read at 450 nm using a microplate reader (Elabscience Co., Ltd., Shanghai, China).

Statistical analysis

SPSS (version 27.0) (IBM, Armonk, New York, USA) was employed for statistical analysis. The data were expressed as the mean ± standard deviation (SD). The t-test was used to check the difference between the data groups with $P < 0.05$ as significant difference.

Results and discussion

Characterization of OMPs

The results showed that the *Escherichia coli* outer membrane proteins (OMPs) demonstrated an estimated molecular weight of 48 kDa using SDS-PAGE with slight deviations from our previous findings (Figure 1), which might be due to the several factors including electrophoresis conditions, post-translational modifications, and underlying protein properties, which were all possible to affect the size of the bands after electrophoresis. The findings of this study were consistent with the previous study that indicated that these factors and other laboratory reasons might affect the size of the bands [38]. It is crucial

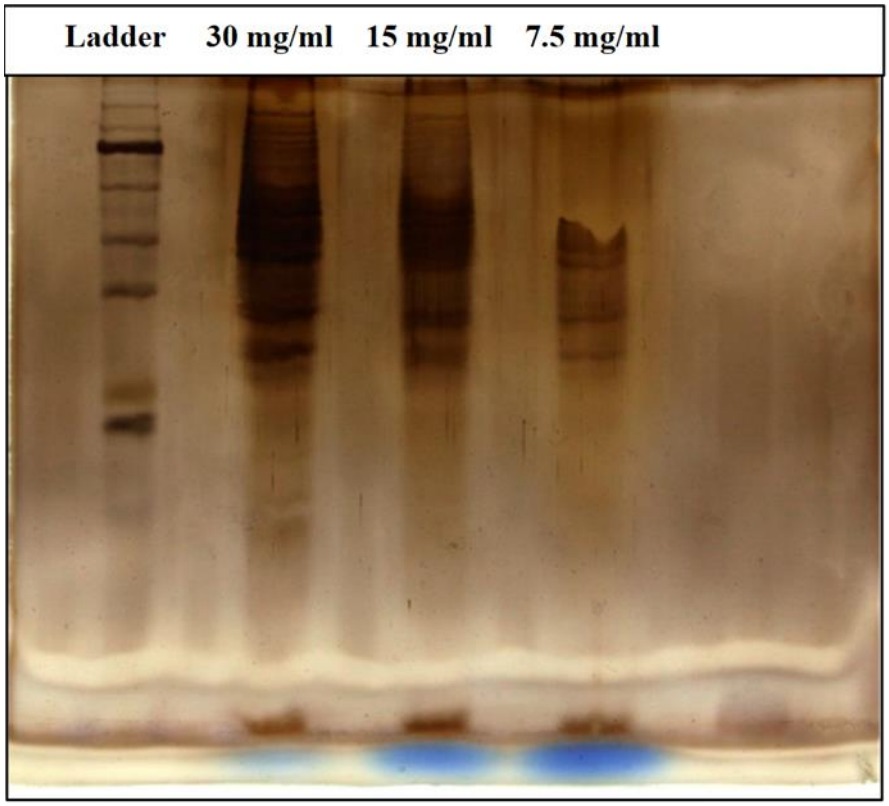

Figure 1. SDS-PAGE of *Escherichia coli* OMPs demonstrated the estimated OMP molecular weight (MW) at 48 KD by comparison with standard MW markers.

to approach molecular weight data interpretation cautiously when relying solely on SDS-PAGE analysis. Several studies have shown a potential correlation between OMP profiles, genetic factors, and bacterial pathogenicity [13-15]. By delving deeper into specific OMP functions and their association with virulence, it may potentially predict bacterial behavior and inform the development of targeted vaccines against *E. Coli* [16-18]. Furthermore, alternative detection methods with heightened sensitivity, cost-effectiveness, and streamlined operation could be more promising for clinical applications [39].

Impact of OMPs on spleen weight index and cell immunity

OMP-producing *Escherichia coli* showed a notable increase in the spleen weight index among experimental animals compared to that in control group. Rabbits exposed to OMPs exhibited a significantly higher spleen weight index of 0.068 ± 0.014 than that of the control group (0.016 ± 0.009), which suggested that OMPs might play a role in inducing spleen enlargement. This finding aligned with prior research, which had consistently demonstrated severe systemic infection and inflammation upon *E. coli* invasion into the host [19-22]. To gain deeper insights, future studies should explore the specific molecular mechanisms connecting OMPs to the observed increase in spleen weight index. By doing so, the key pathways involved in the inflammatory response triggered by these outer membrane proteins should be explored [23]. The increased spleen weight index and histopathological alterations in OMP-exposed rabbits suggested their involvement in triggering inflammatory responses [40]. Delving deeper into the specific mechanisms underlying these observations, including quantifying changes and analyzing affected cell types can provide useful

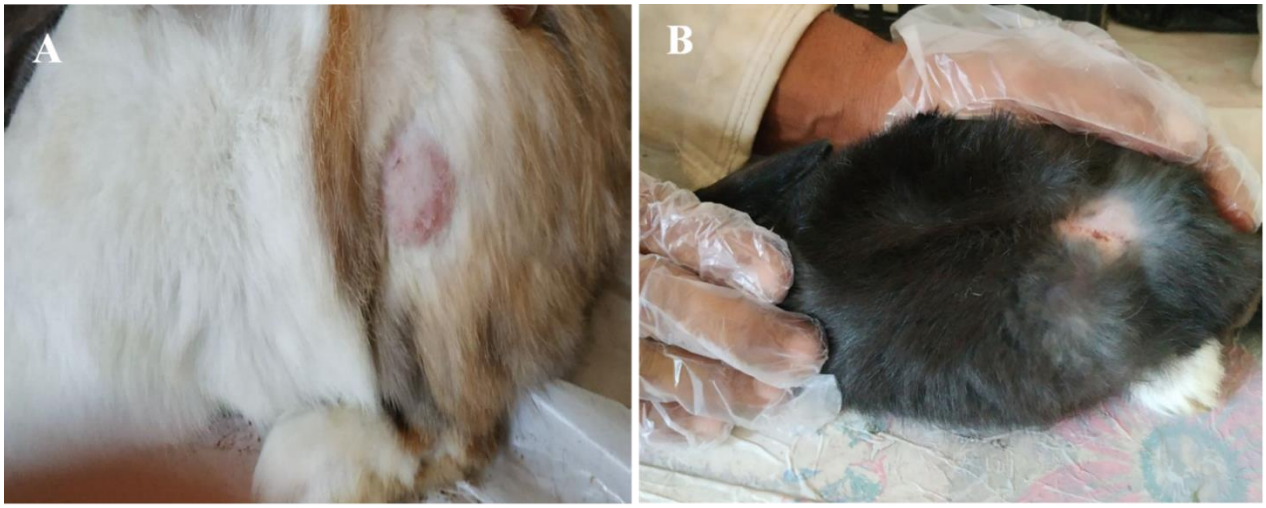

Figure 2. Skin sensitivity of the immunized rabbits with saline (A) and with OMP (B) after 72 hours.

information about the immune response [15, 41]. Furthermore, the reduced bacterial shedding in immunized rabbits' hints at a protective effect mediated by OMPs [10, 32]. While potential strain variations might explain discrepancies with previous studies in terms of shedding duration, future investigations should focus on exploring these strain-specific responses and the immunogenic properties of different OMP variants to optimize their protective potential [42]. This study opened exciting avenues for understanding OMPs' impact on immunity, inflammation, and bacterial shedding. Future research may focus on the exploration of these intricate mechanisms and paving the way for more effective interventions against bacterial pathogens.

Influence of OMPs on skin sensitivity and cell immunity

The results demonstrated that immunized rabbits exhibited reduced bacterial shedding in feces compared to the control group. Fecal bacterial shedding was measured in control and OMP-exposed rabbits over a 72-hour period. The results showed no shedding (00.00 mm) was observed in control group, while rabbits exposed to OMPs displayed a significant increase in shedding at 4 hours as 1.86" ± 0.29 mm and peaking at 24 hours as 10.00" ± 3.00 mm followed by a gradual decrease at 48 hours as 11.66" ± 2.16 mm and 72 hours as 8.68" ± 0.67 mm (Figure 2). This reduction suggested a protective effect mediated by OMPs. Interestingly, our findings diverged from some prior studies regarding the duration of reduced shedding [24-27]. These discrepancies might arise from variations in bacterial strains and their antigenic profiles. Therefore, further investigation into strain-specific responses and the immunogenic properties of different OMP variants is warranted. Additionally, OMPs induced a humoral immune response, characterized by an increase in antibody titer and enhanced binding affinity towards *E. coli* [28, 29]. Furthermore, these proteins facilitated macrophage activation, supporting their potential as vaccine candidates [30]. However, limitations in protection levels necessitated further optimization to maximize their efficacy. Exploring various approaches, such as combining OMPs with adjuvants or targeting specific OMP epitopes, could address these limitations and enhance their protective potential [43].

Cytokine responses to OMP fractions

The results revealed robust responses in IL-10, indicating activation of Th2 immune pathway in OMP-exposed rabbits. The OMP exposure animal group showed a significant increase in IL-10

(105.443 ± 18.942 pg/mL) compared to that in the control group (44.312 ± 5.598 pg/mL), which suggested that OMPs triggered the synthesis of cytokines Th2 (IL-10). These findings aligned with existing reports that associated OMPs with Th2 humoral responses *via* R-LPS residues [31, 32]. To deepen our understanding, future studies should focus on dissecting the specific OMP components responsible for this immune response. Such investigations may unveil valuable insights into the intricate immunomodulatory effects of OMPs.

Cellular inflammatory response

The OMP-LPS group exhibited elevated levels of IL-10 as measured by ELISA. The result heightened cytokine level and suggested the infiltration of inflammatory cells [34]. This finding aligned with the theory that prior exposure to OMP antigens triggered robust inflammatory responses, primarily mediated by macrophages and lymphocytes [34]. Further investigation should explore the specific cell types involved in this infiltration including neutrophils, macrophages, and T lymphocytes. Additionally, delving into the intricate interactions between cytokines and their receptors will provide a more detailed comprehension of the underlying cellular mechanisms [35-37, 44].

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